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(54) Title: DOUBLE RECOMBINANT VACCINIA VIRUS VACCINES			
(57) Abstract <p>Vaccines comprising a recombinant vaccinia virus expressing at least two heterologous genes encoding pathogen antigens are described. A first antigen gene is inserted into the thymidine kinase gene of the vaccinia virus and a second antigen gene is inserted into the hemagglutinin gene of the vaccinia virus. In particular, the hemagglutinin and fusion genes of the rinderpest virus have been inserted into the thymidine kinase and hemagglutinin genes of the vaccinia virus, respectively. Such double recombinant viruses have been found to be highly attenuated while remaining effective in protecting an inoculated host.</p>			
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## DOUBLE RECOMBINANT VACCINIA VIRUS VACCINES

BACKGROUND OF THE INVENTION1. Field of the Invention

5           The present invention relates generally to the field of vaccine preparation and use. More particularly, the present invention relates to recombinant vaccinia virus vaccines capable of expressing antigens from other pathogens.

10           Vaccinia virus is a poxvirus capable of infecting a variety of animal hosts including rabbits, goats, camels, sheep, and cattle. Because of its antigenic similarity to the variola (smallpox) virus, live vaccinia has proven successful in immunizing  
15 individuals against smallpox infection. Indeed, the name vaccine was originally derived from the name vaccinia.

          Recently, there has been widespread interest in the use of recombinant vaccinia virus for vaccination against a variety of viral and other infectious diseases.  
20 Vaccinia is a very large virus having a genome of about 187 kb. About 10% or greater of the genome is either redundant or encodes functions which are not essential for replication. The insertion of heterologous genes into such non-essential regions promises to provide novel  
25 vaccines for numerous diseases. For example, an influenza vaccine has been developed by inserting the influenza hemagglutinin gene into the vaccinia virus thymidine kinase gene. The resulting recombinant vaccinia virus was then used as an immunogen in a vaccine  
30 for protection against influenza. Other examples of successful recombinant vaccinia virus vaccines have also been reported.

          Of particular interest to the present  
invention, recombinant vaccinia virus vaccines have been  
35 developed to immunize against infection by the rinderpest virus. Rinderpest is a highly contagious viral disease

of cattle, buffalo, and other ruminants, and is characterized by high fever, profuse bloody diarrhea, 100% morbidity, and greater than 95% mortality. The rinderpest virus is a member of the family  
5 *Paramyxoviridae* and the genus *Morbilliviruses*. The recombinant vaccines relied on inserting either the hemagglutinin or the fusion gene of the rinderpest virus into the thymidine kinase gene of vaccinia. Cattle vaccinated with either recombinant were protected when  
10 subsequently challenged with lethal doses of rinderpest virus, but certain of the cattle had significant anamnestic response after challenge, indicating replication of the challenge virus. No anamnestic response, however, occurred in cattle vaccinated with a  
15 cocktail of both recombinants. The use of such a cocktail vaccine in the field, however, is cumbersome and expensive and particularly inappropriate in less developed countries where rinderpest is a problem.

Vaccines based on vaccinia virus suffer from  
20 safety problems as a result of the formation of pock lesions at the site of inoculation. The pock lesions can be a source of accidental contamination of the vaccinia virus. In particular, if the lesion is accidentally opened or otherwise compromised, the replicating virus  
25 can be released to the environment where it can infect unintended hosts. Moreover, even highly attenuated vaccinia virus strains can be harmful to immunocompromised individuals, such as patients suffering from acquired immunodeficiency syndrome (AIDS).

30 For these reasons, it would be desirable to provide improved recombinant vaccinia virus vaccines capable of eliciting immunity against a variety of viral and other pathogens. It would be particularly desirable if the vaccinia virus vaccines were able to provide  
35 immunity against subsequent challenge with even very lethal doses of the pathogen while the recombinant vaccinia virus itself is highly attenuated so that it

will produce minimal or no pock formation. Moreover, it would be desirable to provide recombinant vaccinia virus vaccines capable of expressing two or more antigenic genes from the same or different pathogens. In particular, it would be desirable to provide improved vaccines against rinderpest infection which are capable of affording a high degree of immunity with only a single recombinant vaccinia virus strain.

2. Description of the Background Art

Vaccines against rinderpest virus based on recombinant vaccinia virus expressing either the hemagglutinin or the fusion gene of rinderpest are reported in Yilma et al. (1988) Science 242: 1058-1061 and Yilma (1990) Biotechnology 8:1007-1009. The latter reference states applicants' intention to develop a double recombinant vaccinia virus expressing the hemagglutinin and fusion genes of rinderpest for use as a vaccine (page 1008). Perkus et al. (1985) Science 229:981 have prepared a polyvalent recombinant vaccinia virus expressing herpes simplex virus, hepatitis B virus, and influenza virus antigens. While inoculation of rabbits was found to induce a humoral response, no protection against subsequent challenge was demonstrated. Insertional inactivation of the thymidine kinase gene has been shown to further attenuate the Wyeth strain of vaccinia virus (Buller et al. (1985) Nature 317:813). The preparation of recombinant vaccinia virus vaccines is described generally in U.S. Patent No. 4,722,848, the disclosure of which is incorporated herein by reference.

SUMMARY OF THE INVENTION

Vaccines conferring immunity against viral and other pathogens comprise recombinant vaccinia virus having both the thymidine kinase and hemagglutinin genes inactivated, with at least one of said genes being inactivated by insertion of a heterologous gene encoding an exogenous antigen of a pathogen. Usually, the recombinant vaccinia virus will have a first gene

encoding a first antigen inserted into the thymidine kinase gene and a second gene encoding a second antigen inserted into the hemagglutinin gene. Surprisingly, it has been found that such double recombinant vaccinia virus vaccines are capable of conferring a strong protective immunity against the pathogen while being sufficiently attenuated to produce little or no pock lesion. Thus, the vaccines are effective while remaining very safe and unlikely to provide a source for release of the recombinant virus and accidental infection.

The first and second antigen genes will normally be from the same pathogen, but may be from different pathogens. The recombinant vaccinia virus may also express additional heterologous antigen gene(s) from either the same or different pathogens. The present invention requires only that both the thymidine kinase gene and the hemagglutinin gene be inactivated, with at least one of the genes being insertionally inactivated with a heterologous antigen gene.

In a preferred embodiment, the vaccine is a rinderpest vaccine comprising vaccinia virus expressing the hemagglutinin and fusion genes of the rinderpest virus. In a particularly preferred embodiment, the hemagglutinin and fusion genes are inserted into the thymidine kinase and hemagglutinin genes of the vaccinia virus, respectively.

In a first method according to the present invention, a susceptible host is inoculated with a recombinant vaccinia virus to confer immunity against a viral or other pathogen. The vaccinia virus expresses a first heterologous gene encoding a first antigen of a pathogen inserted into the thymidine kinase gene and a second heterologous gene encoding a second antigen of a pathogen inserting into the hemagglutinin gene.

In a second method according to the present invention, a recombinant vaccinia virus is produced by

inserting a first heterologous gene encoding a first viral or other antigen into the thymidine kinase gene and a second heterologous gene encoding a second viral or other antigen into the hemagglutinin gene of the vaccinia virus. The recombinant virus is propagated to produce virus suitable for incorporation into vaccine compositions. Preferably, the vaccinia virus is an attenuated strain, more preferably being the Wyeth strain.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the construction of a vaccinia virus double recombinant strain expressing both the fusion and hemagglutinin genes of the rinderpest virus, as described in more detail in the Experimental section hereinafter.

Fig. 2 is a polyacrylamide gel characterizing the polypeptides expressed by the vaccinia virus double recombinant strain produced in the Experimental section hereinafter.

Fig. 3A is a photograph of the neck region of a cow inoculated with the double recombinant vaccinia virus strain, as described in detail in the Experimental section hereinafter. No pock lesions are apparent.

Fig. 3B is a photograph of vaccinia virus-induced pock lesions in the neck region of a cow inoculated with the cocktail of two single recombinant vaccinia viruses of the same strain, as described in detail in the Experimental section hereinafter.

#### DESCRIPTION OF THE SPECIFIC EMBODIMENTS

According to the present invention, vaccines are provided for conferring immunity against infection by a variety of pathogens, particularly viral pathogens, but also bacteria, spirochetes, protozoa, and the like. The vaccines comprise a recombinant vaccinia virus expressing at least one heterologous gene from at least one pathogen, wherein both the thymidine kinase and hemagglutinin genes of the vaccinia virus have been

inactivated. At least one of the genes will have been inactivated by insertion of the heterologous gene and usually both genes will have been insertionally inactivated, either with the same or different heterologous genes expressing antigens from the same or different pathogens.

The heterologous genes encode exogenous antigens of the pathogen which are capable of eliciting a virus neutralizing (humoral and/or cell-mediated) response when administered to a susceptible host. By exogenous, it is meant that the genes expressing the antigens are not native to the vaccinia strain which is recombinantly modified. Usually, the vaccinia virus will express at least two antigens from a single pathogen, more usually expressing at least two different antigens from the same pathogen. Optionally, the vaccinia virus may express three or more antigens. A list of pathogens with corresponding antigens is provided in Table 1.

TABLE 1

	<u>Pathogen</u>	<u>Antigen(s)</u>	<u>Reference</u>
5	Rinderpest virus	Hemagglutinin Fusion	Yilma et al. (1988) Science 242:1058-1061.
10	Hepatitis B virus	Surface Antigen	Smith et al. (1983) Nature 302:490.
15	Influenza virus	Hemagglutinin (HA) PB1 PB2 PA NP M1 NS1	Panicali et al. (1983) PNAS USA 80:5364; Flexner et al. (1988) Nature 335:229; Yewdell et al. (1985) PNAS USA 82:19; Smith et al. (1987) Virol. 160:336; Smith et al. (1983) PNAS USA 80:7155; Coupar et al. (1986) J. Immunol. 16:1479; and Townsend et al. (1988) J. Exp. Med. 168:1211.
20			
25			
30			
35	Herpes simplex virus	Glycoprotein D (gD) Thymidine kinase (TK) Glycoprotein B Glycoprotein G	Paoletti et al. (1984) PNAS USA 81:193; Panicali et al. (1982) PNAS USA 79:4927; and McLaughlin- Taylor et al. (1988) J. Gen. Virol. 62:1731; and Sullivan and Smith (1989) J. Gen. Virol. 68:2587.
40			
45			
50	<i>Plasmodium knowlesi</i>	Sporozoite	Smith et al. (1984) Science 224:397.

5	Vesicular stomatitis virus	G protein N protein M protein	Mackett et al. (1985) Science 227:433; and Li et al. (1988) J. Virol. 62:776.
10	Human immuno- deficiency virus	gp160 gag/pol gene products	Chakrabarti et al. (1986) Nature 320:535; and Flexner et al. (1988) J. Virol. 166:339.
15	Human papilloma virus	L1 gene product	Browne et al. (1988) J. Gen. Virol. 69:1263.
20	Human cytomegalovirus	Glycoprotein B	Cranage et al. (1986) EMBO J. 5:3057.
25	Epstein-Barr virus	gp340/220	Mackett et al. (1985) EMBO. J. 4:3229; and Morgan et al. (1988) J. Med. Virol. 25:189.
30	Pseudorabies	gp50	Marchioli et al. (1987) J. Virol. 61:3977.
35	Measles virus	Hemagglutinin fusion	Drillien et al. (1988) PNAS USA 85:1252.
40	<i>Plasmodium falciparum</i>	CSP S-antigen	Langford et al. (1986) Mol. Cell Biol. 6:3191.

45 In a preferred embodiment, first and second  
heterologous genes encoding the desired antigens will be  
inserted into the thymidine kinase and hemagglutinin  
genes of the vaccinia virus, respectively. It has been  
found that the resulting inactivation of both the  
thymidine kinase and hemagglutinin genes results in  
50 substantial attenuation of the vaccinia virus without  
significant loss of the ability of the virus to elicit  
virus neutralizing antibodies and protect an inoculated

host against subsequent challenge. The location of the thymidine kinase gene and the hemagglutinin gene on the vaccinia virus genome is illustrated in Fig. 1 which depicts a *HindIII* digest of the vaccinia virus genome (Wyeth strain), wherein thymidine kinase is in the J region and hemagglutinin is in the A region. The thymidine kinase gene is further described in Buller et al. (1985) *Nature* 317:813-815 and the hemagglutinin gene in Shida (1986) *Virology* 150:451-462. Both of these references are incorporated herein by reference.

Genes encoding for the desired antigens can be isolated from the pathogens using conventional techniques. In the case of RNA viruses, cDNA copies of the genes can be made.

Suitable vectors for inserting the antigen genes into the thymidine kinase and hemagglutinin genes of vaccinia virus are described in the scientific literature. A particularly suitable vector for inserting antigen genes into the thymidine kinase gene of vaccinia is pGS53 described in Mackett et al. (1985) *Science* 227:433-435, while a vector for inserting antigen genes into the hemagglutinin gene of vaccinia is pVY6 described in Flexner et al. (1988) *Nature* 335:259-262. The disclosures of both of these references are incorporated herein by reference. Incorporation of genetic material into these vectors and the use of these vectors for inserting the genetic material into vaccinia virus is well within the skill of the art. Standard procedures for constructing vaccinia virus recombinants are described in Chakrabarti et al. (1985) *Mol. Cell Biol.* 5:3403 and U.S. Patent No. 4,722,848, the disclosures of which is incorporated herein by reference.

The vaccinia virus which is modified by insertion of the antigen genes will preferably be an attenuated strain, i.e., having reduced virulence compared with wild type vaccinia virus. Suitable attenuated vaccinia virus strains include Wyeth (New York

City Board of Health), Lister, and the like. Particularly preferred is the use of the Wyeth strain, available from Flow Laboratories, McLean, VA.

5 The construction of an exemplary double recombinant vaccinia virus expressing the hemagglutinin and fusion genes of the rindepest virus is described in detail in the Experimental section hereinafter. The hemagglutinin and fusion genes of rinderpest are inserted into the thymidine kinase and hemagglutinin genes of the  
10 Wyeth (NYCBH) strain of vaccinia virus, respectively. The resulting double recombinant virus was found to be highly attenuated and in particular was found to cause little or no pock lesion formation when inoculated into cattle and other hosts. The double recombinant virus,  
15 however, was found to be a very effective vaccine and provided protection of cattle against highly lethal doses of the rinderpest virus. It is believed that the exemplary double recombinant vaccinia virus will also afford protection against other *Morbilliviruses* which are  
20 antigenically similar to rinderpest, including the peste-des-petits ruminants (PPR) virus, the measles virus, the canine distemper virus, and the *Morbillivirus* of seals.

The recombinant vaccinia virus may be propagated in cell culture or by scarification of  
25 susceptible hosts, such as calves, sheep, or horse, and the like. Methods for isolating and purifying the virus produced from both cell culture and animal sources are well known in the art.

Vaccine compositions according to the present  
30 invention will incorporate the live attenuated double recombinant vaccinia viruses prepared as described above. The vaccinia virus may be incorporated in a physiologically-acceptable medium, such as water, saline, phosphate buffered saline, and may be administered in any  
35 conventional manner, including subcutaneously, intradermally, orally, or nasally, and the like. The preparation and administration of such vaccine

compositions are described in numerous standard references, such as *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, PA, 16th Ed., 1982, the disclosure of which is incorporated herein by reference.

5 The preparation of veterinary vaccines is described in Tizard, *An Introduction to Veterinary Immunology*, 2nd. Ed., 1982, the disclosure of which is incorporated herein by reference.

10 In veterinary applications, the vaccines of the present invention may frequently be lyophilized and administered by scarification. Scarification procedures are well known in the art and described in standard references, such as Quinnan, ed., *Vaccinia Virus as Vector for Vaccine Antigens*, Elsevier-North Holland,

15 1985, the relevant disclosure of which is incorporated herein by reference.

The dosage form and virus content of the vaccine will vary depending on the nature of the host and the disease. For injectable vaccines, a single dose will

20 usually have a total volume including carrier, and any other components, in the range from about 0.05 ml to about 0.1 ml. The amount of virus in each dose will usually be in the range from about  $10^2$  to  $10^8$  pfu.

The number and temporal spacings of the

25 inoculations will be sufficient to elicit the desired immunoprotective response against subsequent challenge by the pathogen or pathogens. There will be at least one inoculation, and in many cases at least two inoculations spaced at least one month apart. In many cases, a final

30 inoculation may be administered at some longer interval following initial series of administrations. The selection of optimum administration patterns for a particular vaccine formulation is well within the skill of the art.

35 The following examples are offered by way of illustration, not by way of limitation.

## EXPERIMENTAL

### Materials and Methods

#### Cells and Viruses

Vero, human 143 TK<sup>-</sup>, and CV-1 cells were  
5 propagated in Dulbecco's modified Eagle's medium (DMEM)  
supplemented with heat-inactivated fetal bovine serum.  
For the propagation of TK<sup>-</sup> cells, DMEM was supplemented  
with 5-bromodeoxyuridine (BrdUrd) at 25 ug/ml. The  
10 highly pathogenic Kabete "O" strain of rinderpest virus  
(RPV) was propagated in Vero cells and used in all  
studies. The Wyeth strain of vaccinia virus (VV; clone  
B-3-1), obtained from Flow Laboratories (McLean, VA) was  
used exclusively for the generation of recombinants. All  
15 VV recombinants were propagated in Vero cells according  
to the guidelines of the Animal and Plant Health  
Inspection Service of the United States Department of  
Agriculture (USDA-APHIS) and the Office Internationale de  
Epizooties (OIE).

#### Recombinant Plasmids

20 Full-length cDNAs coding for the hemagglutinin  
(H) and fusion (F) proteins of RPV were cloned as  
described previously (Yamanaka et al. (1988) Virology  
166:251-253 and Hsu et al. (1988) Virology 166:149-153).  
Plasmid vectors pVY6 (Flexner et al. (1988) Nature  
25 335:259-262) and pGS53 (Mackett et al. (1985) Science  
227:433-435) were utilized for the construction of VRV-FH,  
the vaccinia virus double recombinant expressing both the  
F and the H genes of RPV. Plasmid vectors pVY6 and pGS53  
direct the cloning of genes in the hemagglutinin (HA) and  
30 thymidine kinase (TK) regions of the vaccinia virus  
genome, respectively.

#### Radioimmunoprecipitation

Proteins expressed by VV recombinants were  
characterized by radioimmunoprecipitation as previously  
35 described (Grubman et al. (1988) Virology 163:261-267).  
Rabbit anti-measles H was used for specific  
immunoprecipitation of the H protein of RPV. Rabbit

antiserum directed against the carboxy terminus of measles virus F protein was used for the immunoprecipitation of the F protein of RPV. Except for the first amino acid, the 18 amino acid long peptide used for generation of the antisera was completely conserved between measles virus and RPV (Vialard et al. (1990) J. Virol. 64:37-50 and Hsu et al. (1988), *supra.*).

#### Immunization Studies in Cattle

Protective immune response studies were conducted in yearling cattle in the high containment facility at the Plum Island Animal Disease Center (USDA-APHIS) according to proper institutional guidelines. Cattle used in these experiments were shown to be seronegative to RPV and vaccinia virus by serum neutralization (SN) and plaque reduction assays, respectively. One contact animal (#111), however, had cross-reacting antibody to RPV which was not protective (Table 2). Cattle were vaccinated with  $10^8$  pfu of VV recombinants by intradermal inoculation and scarification in the neck region. In addition, contact animals were housed with vaccinates in order to test for transmission of VV recombinants from vaccinated to nonvaccinated groups of animals. On the day of challenge, one additional cow was brought in as a fresh control to assure the presence of an animal susceptible to RPV, in case of VV recombinants had been transmitted to contact animals. For the determination of protective immunity, all cattle were challenged with  $10^3$  TCID<sub>50</sub> of RPV one month postvaccination (Yilma et al. (1988) Science 242:1058-1061). It has been demonstrated previously in a study using 19 animals, that as little as one TCID<sub>50</sub> administered subcutaneously in the prescapular lymph node region induced clinical rinderpest with 100% mortality (Yilma et al. (1988), *supra.*).

## RESULTS

### Construction of VV Recombinants

The strategy for the construction of vRVFH, the vaccinia virus double recombinant expressing both the F and H genes of RPV, is outlined in Fig. 1. First, vRVF, the single recombinant expressing the F gene from the HA region of the vaccinia virus genome, was constructed. Briefly, the F cDNA of RPV was excised from plasmid pVRVF6 (Yilma et al. (1988) *supra.*) by digestion with EcoR1, filled in with the Klenow polymerase, and then cloned in the SmaI site of plasmid vector pVY6 to generate pVYRVF. The F gene was then cloned in the HA region of vaccinia virus by homologous recombination between pVYRVF and the Wyeth strain of vaccinia virus in CV-1 cells. Recombinants expressing the F gene (vRVF) were selected by their blue phenotype in the presence of X-gal. In preparation for the construction of vRVFH (double recombinant), the H gene of RPV was excised from plasmid pRVH6 by digestion with EcoR1 (Yilma et al. (1988) *supra.*). The fragment was then blunted with Klenow polymerase and cloned in the SmaI site of the vaccinia virus shuttle vector pGS53 to generate pGSRVH. The double recombinant (vRVFH) was generated by homologous recombination between pGSRVH and vRVF in CV-1 cells (Mackett et al. (1985), *supra.*). TK<sup>-</sup> vaccinia viruses were picked by BrdUrd selection, and double recombinants were distinguished from contaminating, spontaneous TK<sup>-</sup> mutants by plaque hybridization with H cDNA of RPV.

### Expression of F and H Proteins of RPV by VV Recombinants

The expression of authentic F and H proteins by vRVFH, F by vRVF, and H by vRVH was demonstrated by specific immunoprecipitation (Fig. 2). Monolayers of human 143 TK<sup>-</sup> cells were infected with 10 pfu/cell of vaccinia virus in medium containing no methionine or cysteine. After 2 h at 37°, 100 µCi of [<sup>35</sup>S]methionine + cysteine (translab, Amersham) was added and the

incubation was continued for overnight (16 h). A mixture of anti-F and anti-H antibodies to measles virus was used to precipitate polypeptides from infected cells. The preparation of cytoplasmic extracts, immunoprecipitation, and polyacrylamide gel electrophoresis were performed as previously described (Varsanyi et al. (1987) J. Virol. 61:3896). Lane 1: Cells infected with vaccinia virus double recombinant (vRVFH) expressing both the F and H genes of RPV. Lane 2: Cells infected with vaccinia virus single recombinant (vRVF) expressing the F gene of RPV. Lane 3: Cells infected with vaccinia virus single recombinant (vRVH) expressing the H gene of RPV. Lane 4: Wild type vaccinia virus (Wyeth). Lane 5: Molecular weight markers (kDa). Fluorographs revealed that cells infected with vRVFH expressed both the F and the H proteins of RPV of the expected size and MW. Similarly, the F protein expressed by vRVF and the H by vRVH were of the correct size suggesting that the extent of glycosylation is similar to that occurring in cells infected with RPV (Grubman et al. (1988), *supra.*).

#### Immunization Studies in Cattle

Two groups of cattle were vaccinated with VV recombinants in separate isolation rooms. In the first group, five animals were vaccinated with vRVFH (Table 2).

Table 2

SN titers of cattle vaccinated with vaccinia virus double recombinant (vRVFH) expression the F and H genes of RPV.

	Cow #	Day 0	Day 8	Day 14	Day 21	Day 28	Day 42
5	102	-	-	-	-	1	Dead
	111	16	12	6	12	6	Dead
	122	0	0	0	2	6	Dead
10	112	2	64	16	64	48	256
	113	0	32	24	48	24	32
	126	0	128	48	64	24	<100
	134	0	48	64	64	96	96
	135	0	12	64	32	64	64

15 Cow #102 was a control animal that was included in the group on the day of challenge (day 28). Animals #111 and #122 were unvaccinated, contact controls. The rest of the animals were vaccinated on day 0 with  $10^8$  pfu of vRVFH, and serum samples were taken weekly during the course of the experiment. SN titers were determined by the prevention of the cytopathic effects of 100 TCID<sub>50</sub> of RPV in Vero cells. On day 28, all animals were challenged with 1000 TCID<sub>50</sub> of the pathogenic strain of Kabete "0" RPV.

20

25

In the second group, four animals were vaccinated with a cocktail of vRVF and vRVH (Table 3).

Table 3

SN titers of cattle vaccinated with a cocktail of vaccinia virus single recombinants expressing the F (vRVF) and H (vRVH) genes of RPV.

	Cow #	Day 0	Day 8	Day 14	Day 21	Day 28	Day 42
	121	0	0	0	0	0	Dead
	133	0	3	0	1	0	Dead
5							
10	101	0	24	24	96	128	256
	118	0	128	64	24	12	64
	124	0	24	32	64	96	64
	131	0	96	384	32	128	64

15 Animals #121 and #133 were unvaccinated, contact controls. The rest of the animals were vaccinated on day 0 with  $10^8$  pfu of vRVF+vRVH, and serum samples were taken weekly during the course of the experiment. SN titers were determined by the prevention of the cytopathic effects of 100 TCID<sub>50</sub> of RPV in Vero cells. On day 28, 20 all animals were challenged with 1000 TCID<sub>50</sub> of the pathogenic strain of Kabete "0" RPV.

25 In addition, two unvaccinated animals were included in each group in order to assess the transmissibility of VV recombinants from vaccinated to contact animals.

30 Pock lesions developed as early as four days in cattle vaccinated with the cocktail (Fig. 3B). The lesions were limited to the site of inoculation and were healed completely by two weeks postvaccination. In contrast, animals vaccinated with vRVFH developed very tiny or no detectable lesions (Fig. 3A). Cow #126 (Fig. 3) was vaccinated with vRVFH (double recombinant); 35 note the lack of detectable pock lesions at the site of inoculations (Fig. 3A). Cow #118 was vaccinated with vRVF+vRVH (Cocktail) and developed pock lesions at the two sites of inoculation (see arrows) (Fig. 3B).

A thorough examination failed to demonstrate pock lesions in the contact animals in both groups. Further, serum samples taken on the days of vaccination (day 0) and challenge (day 28) were negative to vaccinia virus by SN and plaque reduction assays (data not shown).  
5 All animals vaccinated with VV recombinants produced SN antibodies to RPV (Tables 2 and 3). One month post vaccination, all animals in both groups were challenged with  $10^3$  TCID<sub>50</sub> of the pathogenic Kabete "0" strain of RPV.  
10 Cattle vaccinated with VV recombinants (both groups) were completely protected from rinderpest, exhibiting no detectable illness, and a normal temperature of 38°C. The four unvaccinated contacts (including #111 with the cross-reactive antibody to RPV) and the one control  
15 animal developed high fever (42°C) by day two and died by day six after challenge. They also developed lesions typical of severe rinderpest, characterized by sloughing and erosion of the epithelial lining of the gastrointestinal tract and bloody diarrhea. After daily  
20 monitoring for two weeks and a lack of detectable clinical disease in vaccinated animals, the experiment was terminated.

All cattle vaccinated with the recombinants produced SN antibody to RPV as early as 8 days after  
25 vaccination. However, all contact and control animals lacked detectable SN antibody to RPV during the course of the experiment.

Although the foregoing invention has been described in detail for purposes of clarity of  
30 understanding, it will be obvious that certain modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A vaccine for conferring immunity against a pathogen, said vaccine comprising recombinant vaccinia virus having both the thymidine kinase gene and the hemagglutinin gene inactivated and expressing at least one exogenous antigen of the pathogen, wherein a first gene expressing the exogenous antigen is inserted into at least one of the thymidine kinase gene and the hemagglutinin gene of vaccinia virus.
2. A vaccine as in claim 1, wherein the recombinant vaccinia virus is present in an physiologically acceptable carrier in an amount effective to elicit viral neutralizing activity against the pathogen when administered to a susceptible host.
3. A vaccine as in claim 1, wherein the recombinant vaccinia virus is lyophilized.
4. A vaccine as in claim 1, wherein the pathogen is a virus.
5. A vaccine as in claim 4, wherein the recombinant vaccinia virus expresses a second gene encoding a second exogenous antigen, said second gene being inserted into the other of the thymidine kinase gene and the hemagglutinin gene.
6. A vaccine as in claim 5, wherein the first and second genes encode different antigens of the same virus.
7. A vaccine as in claim 5, wherein the first gene is the hemagglutinin gene of rinderpest virus and the second gene is the fusion gene of rinderpest virus.

8. A vaccine for conferring immunity against rinderpest virus, said vaccine comprising recombinant vaccinia virus expressing the fusion and hemagglutinin genes of rinderpest, wherein at least the thymidine kinase gene and the hemagglutinin gene of the vaccinia virus have been inactivated.

9. A vaccine as in claim 8, wherein the hemagglutinin and fusion genes of rinderpest virus have been inserted into the thymidine kinase and hemagglutinin genes of vaccinia virus, respectively.

10. A vaccine as in claim 8, wherein the recombinant vaccinia virus is present in an physiologically acceptable carrier in an amount effective to elicit viral neutralizing activity against the pathogen when administered to a susceptible host.

11. A vaccine as in claim 8, which has been lyophilized.

12. A vaccine as in claim 8, wherein the recombinant virus is derived from an attenuated strain of vaccinia.

13. A vaccine as in claim 12, wherein the attenuated strain is Wyeth.

14. Recombinant vaccinia virus having a first heterologous gene encoding a viral antigen inserted into the thymidine kinase gene and a second heterologous gene encoding a viral antigen inserted into the hemagglutinin gene.

15. Recombinant vaccinia virus as in claim 14, wherein first and second heterologous genes encode different viral antigens.

16. Recombinant vaccinia virus as in claim 15, wherein the first and second heterologous genes comprise the hemagglutinin and fusion genes of rinderpest.

5 17. Recombinant vaccinia virus as in claim 16, wherein the first heterologous gene is the hemagglutinin gene and the second heterologous gene is the fusion gene.

10 18. Recombinant vaccinia virus as in claim 14, wherein has been lyophilized.

15 19. Recombinant vaccinia virus as in claim 14, wherein the recombinant virus is derived from an attenuated strain of vaccinia.

20 20. Recombinant vaccinia virus as in claim 19, wherein the attenuated strain is Wyeth.

25 21. A method for vaccinating a susceptible host to confer immunity against a viral pathogen, said method comprising inoculating the host with a recombinant vaccinia virus having both the thymidine kinase gene and the hemagglutinin inactivated and a first heterologous gene encoding a first antigen of the pathogen inserted into at least one of the thymidine kinase gene and the hemagglutinin gene.

30 22. A method as in claim 21, wherein a second gene encoding a second exogenous antigen is inserted into the other of the thymidine kinase gene and the hemagglutinin gene.

35 23. A method as in claim 22, wherein the first and second heterologous genes encode different viral antigens.

24. A method as in claim 23, wherein the first and second heterologous genes comprise the hemagglutinin and fusion genes of rinderpest.

5           25. A method as in claim 24, wherein first heterologous gene is the hemagglutinin gene and the second heterologous gene is the fusion gene.

10           26. A method as in claim 24, wherein the recombinant vaccinia virus is present in a physiologically acceptable carrier in an amount effective to elicit viral neutralizing activity against the pathogen when administered to a susceptible host.

15           27. A method as in claim 21, wherein the recombinant vaccinia virus has been lyophilized.

20           28. A method as in claim 21, wherein the recombinant virus is derived from an attenuated strain of vaccinia.

          29. A method as in claim 28, wherein the attenuated strain is Wyeth.

25           30. A method for producing a recombinant vaccinia virus suitable for use as a vaccine, said method comprising:

          inserting a first heterologous gene encoding a first antigen into the thymidine kinase gene of a  
30   vaccinia virus;

          inserting a second heterologous gene encoding a second antigen into the hemagglutinin gene of the vaccinia virus; and

          propagating the resulting recombinant virus.  
35

          31. A method as in claim 30, wherein the vaccinia virus is an attenuated strain.

32. A method as in claim 31, wherein the attenuated strain is Wyeth.

5 33. A method as in claim 32, wherein the first gene is the hemagglutinin gene of rinderpest virus and the second gene is the fusion gene of rinderpest virus.

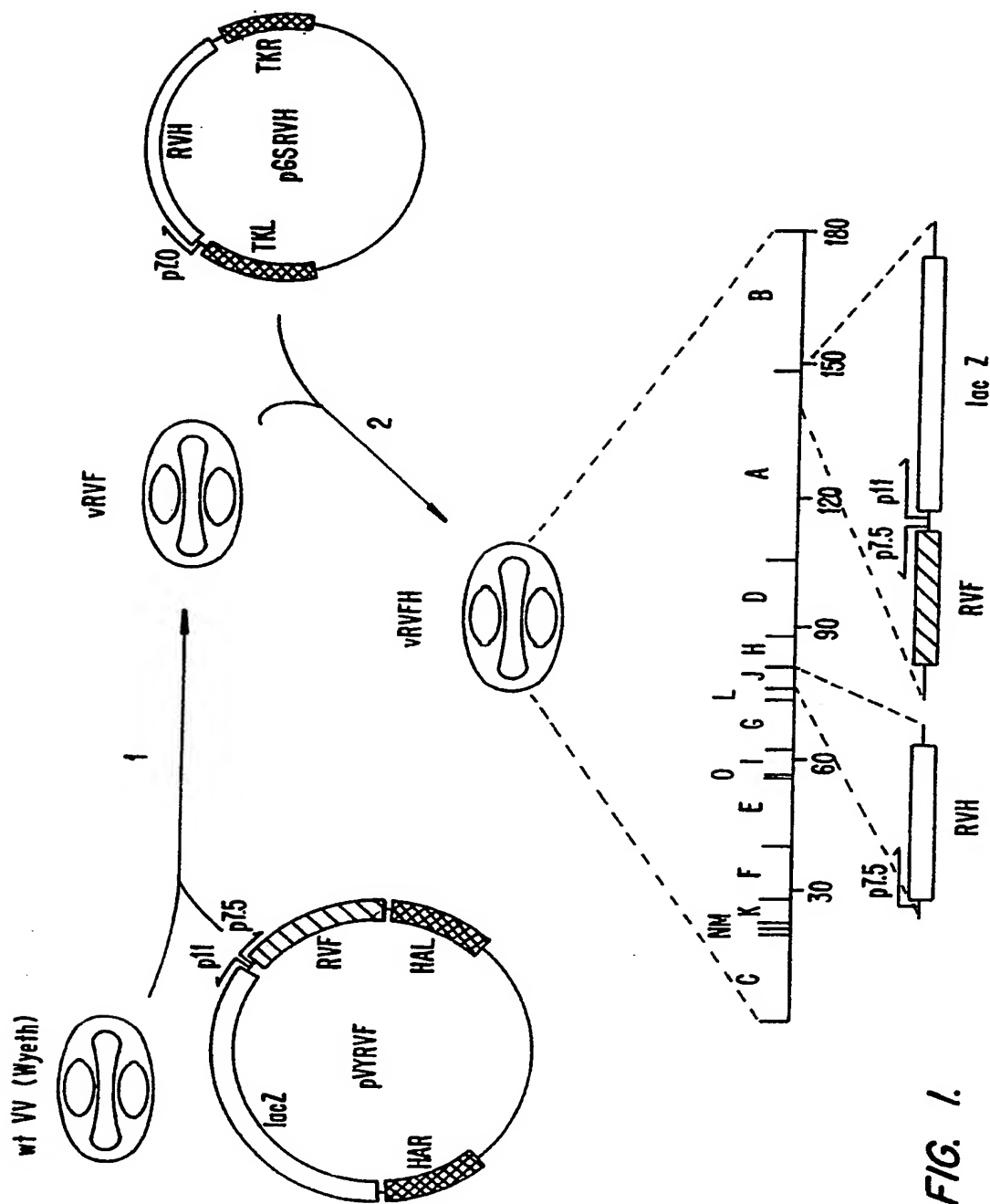


FIG. 1.

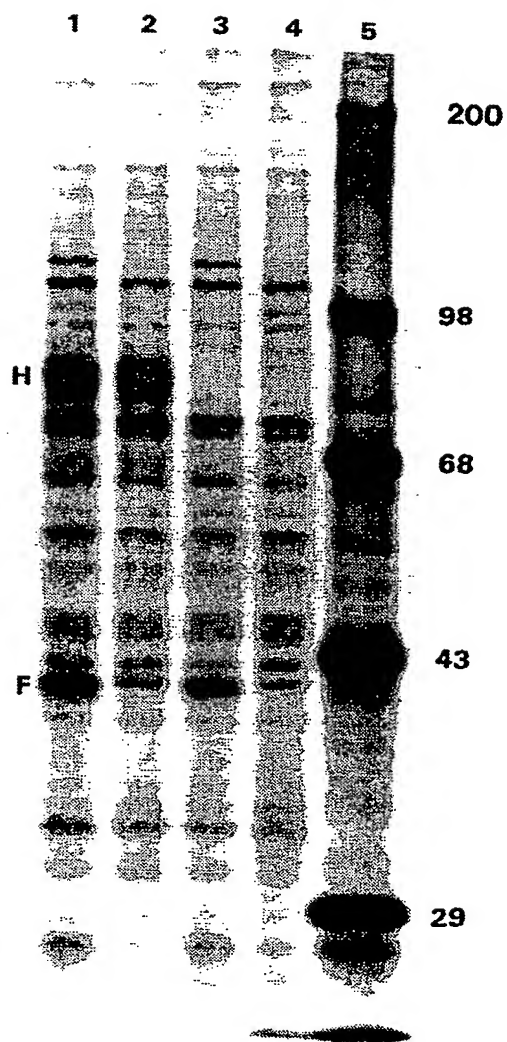


FIG. 2.



**FIG. 3A.**



**FIG. 3B.**

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/04749

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 39/12

US CL : 424/89

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/89

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

search terms: vaccinia virus, recombinant, rinderpest virus, vaccine

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, volume 317, issued 31 October 1985, Buller et al, "Decreased virulence of Recombinant Vaccinia Virus Expressin Vectors is Associated With a Thymidine Kinase-Negative Phenotype", pages 813-815, see entire article.	1-6,21-33
Y	Science, volume 229, issued 06 September 1985, Perkus et al., "Recombinant Vaccinia Virus: Immunization Against Multiple Pathogens", pages 981-984, see entire article.	1-6,21-33
Y	Science, volume 242, issued 18 November 1988, Yilma et al, "Protection of Cattle Against Rinderpest With Vaccinia Virus Recombinants Expressing The HA Or F Gene", pages 1058-1061, see entire article.	7-20

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

10 August 1992

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Washington, D.C. 20231

Authorized officer

LYNETTE F. SMITH

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

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